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Proinsulin Biosynthesis in Broken-Cell Preparations of Islets of Langerhans

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1. Rabbit islets of Langerhans were disrupted by ultrasonic methods and the sonicated preparations were used to study proinsulin biosynthesis. 2. When [^{3}H]leucine is incubated in such preparations, incorporation takes place into proinsulin, as evidenced by characterization on polyacrylamide gels, and by the conversion of this labelled material into insulin, by using trypsin. 3. The labelled proinsulin may also be purified by anti-insulin antibody bound to Sepharose. 4. With the broken-cell preparation it was shown that incorporation of leucine is accelerated by increasing the glucose content of the medium from 2mM to 16mM. However, 16mM-galactose or -sucrose did not stimulate incorporation significantly from basal values. This effect of glucose was abolished by cycloheximide. 5. The significance of these findings in relation to the mechanism of glucose stimulation of proinsulin biosynthesis is discussed.

The stimulatory effects of glucose on the biosynthesis of insulin have been the subject of detailed studies, originally in pancreas slices (Taylor, 1964; Parry & Taylor, 1966), and later in isolated islets of Langerhans (Howell & Taylor, 1968*a*; Steiner *et al.*, 1969; Lin & Haist, 1969; Morris & Korner, 1970; Permutt & Kipnis, 1972; Ashcroft *et al.*, 1976). There is, however, no clear view as to how glucose produces this effect, although it has been suggested that the effect is perhaps mediated at both a translational level as well as a transcriptional one (Permutt & Kipnis, 1972). Whether the stimulatory response requires glucose to be metabolized or whether it is a consequence of its interaction with a membrane receptor is still uncertain.

As a first step to understanding these effects of sugars, islets have been ultrasonically disrupted, to decide whether glucose may still show a stimulatory effect when the β-cell structure is disrupted. In the experiments described below, the effects of glucose and other sugars on biosynthesis are examined in a broken-cell preparation of islets and in a simple cell-free system. A preliminary account of some aspects of this work has appeared (Parry & Taylor, 1975).

Materials and Methods

Animals

Male New Zealand White rabbits were obtained from Redfern Animal Breeders, Blenchley, Kent, U.K. Each animal weighed 2.5–3.0kg.

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Reagents

Collagenase was obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K., and L-[4,5-^{3}H]leucine (sp. radioactivity 30–50Ci/mmol) from The Radiochemical Centre, Amersham, Bucks., U.K. CNBr-activated Sepharose 4B and Sephadex G-50 (fine grade) were from Pharmacia Fine Chemicals, London W.5, U.K. Soluene, 2,5-diphenyloxazole (PPO) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (dimethyl-POPOP) were obtained from Packard Instruments Co., Caversham, Berks., U.K. ATP, GTP, phosphocreatine and creatine kinase were obtained from Boehringer Corp., Lewes, Sussex, U.K. All other chemicals were of analytical grade from BDH Chemicals, Poole, Dorset, U.K. The anti-(ox insulin) antiserum was produced, at University of Sussex, in guinea pigs by the methods described by Taylor *et al.* (1965). Crystalline ox insulin was a generous gift from Burroughs Wellcome, Dartford, Kent, U.K. Proinsulin was kindly provided by Eli Lilly, Indianapolis, IN, U.S.A.

Incubation of broken-cell and cell-free preparations of islets

For each determination, 100 islets were prepared by the collagenase digestion of rabbit pancreas, by the method of Howell & Taylor (1968*b*). The islets were preincubated for 20min at 37°C in 0.2ml of bicarbonate buffer, pH7.4 (Gey & Gey, 1936), containing 2mM-glucose, which had been gassed previously with O_2/CO_2 (19:1). The incubation medium was removed after gentle centrifugation

(50*g* for 5min). The islets were then suspended in 0.2ml of a Tris buffer and subjected to ultrasonic disruption (1MHz for 2s). The buffer contained 80mM-KCl, 25mM-Tris/HCl and 4mM-magnesium acetate, pH7.5. Each ml of this medium contained 5μM-ATP, 1μM-GTP, 3.3μM-phosphocreatine, 1unit (40μg) of creatine kinase and amino acids at a concentration of 1μM for all 19 amino acids except for leucine. The sonicated preparation was then added to 10μCi of L-[^{3}H]leucine, together with the glucose or other sugars, in an incubation vial, and this mixture was further incubated for 1h at 37°C in a shaking water bath.

In other experiments a crude cell-free preparation of islets was made by centrifuging the sonicated preparation at 900*g* for 5min. The supernatant (170μl) was removed, and added to 10μCi of [^{3}H]-leucine. This mixture was then incubated for 1h at 37°C.

In each case, immediately after the incubation the proteins were precipitated with 10% (w/v) trichloroacetic acid in the presence of 1mg of bovine plasma albumin, and washed with 5% (w/v) trichloroacetic acid.

Gel filtration of sonicated preparation

The sonicated preparation was mixed with 250μl of 1M-acetic acid and the whole applied to a column (80cm × 1cm) of Sephadex G-50 (fine grade) that had previously been equilibrated with 1% albumin in 1M-acetic acid. Labelled material was eluted with 1M-acetic acid, and fractions (1ml) were collected and assayed for radioactivity in a Beckman LS233 liquid-scintillation counter. The peaks of radioactivity corresponding to the elution position of proinsulin were taken and freeze-dried in the presence of 1mg of albumin. In the cell-free experiment, gel filtration was omitted. The efficiency of radioactivity counting was 42%.

Immunobinding of proinsulin

Further purification of the proinsulin was accomplished by using an anti-insulin serum bound to Sepharose (AIS–Sepharose) by the method of Berne (1975). The trichloroacetic acid precipitate or freeze-dried material was taken up in 100μl of 0.01M-HCl and made up to 1ml with phosphate-buffered saline (0.9% NaCl/5mM-sodium phosphate), pH7.4. To this was added 15mg of AIS–Sepharose and bovine plasma albumin, to give an albumin concentration of 0.5%. The mixture was then incubated at room temperature (18°C) in a rotary mixer at 20rev./min for 2h. After centrifugation (1000*g* for 15min) the Sepharose was then washed six times with phosphate-buffered saline before being extracted with acid/ethanol (ethanol/water/conc. HCl, 150:50:3, by vol.).

Determination of radioactivity

A portion of the extract was taken for immunoassay of insulin by the method of Hales & Randle (1963), with an ox insulin standard. At the same time, 200μl was also pipetted into 2ml of a Triton-based scintillator (300ml of Triton X-100, 700ml of toluene, 4g of PPO) for determination of radioactivity. Results are expressed as radioactivity present in insulin. No attempt has been made to distinguish between label incorporated separately into insulin and proinsulin in these experiments. The counting efficiency was 32%.

Polyacrylamide-gel electrophoresis

The product was further identified by polyacrylamide-gel electrophoresis by a method based on that of Davis (1964), with a 9% gel with 2% cross-linkage and a 5mM-Tris/glycine buffer, pH8.9. A further portion of the acid/ethanol extract from AIS-Sepharose was freeze-dried, and taken up in 50μl of 0.01M-HCl and made up to a final volume of 200μl with the Tris/glycine buffer, containing 100μg of ox insulin carrier. Pig proinsulin and ox insulin were also run as suitable markers. After electrophoresis for 90min, the marker gel was stained with Coomassie Blue, and appropriate (3mm) segments were dissolved in 0.2ml of 100-volume H_2O_2 at 60°C for 8h. Samples were assayed for radioactivity in a scintillant containing 500mg of dimethyl-POPOP and 4g of PPO/litre of toluene.

To obtain further evidence for the identity of proinsulin, a sample of the acid/ethanol extract from antibody-treated material was subjected to enzyme hydrolysis with dicyclohexylcarbodi-imide-treated trypsin, as described elsewhere (Parry & Taylor, 1974), and the hydrolysed material dialysed in the presence of 100μg of ox insulin against water, and then freeze-dried. The dried material was then subjected to polyacrylamide-gel electrophoresis, and radioactivity determined in segments of the gel as above. The counting efficiency was 30%.

Results

Characterization of proinsulin in sonicated preparations of islets

Fig. 1 shows the results of gel filtration of the products formed when an islet homogenate was incubated with [^{3}H]leucine. The major peak of higher-molecular-weight material had the elution characteristics of proinsulin (see Parry & Taylor, 1974), although, in crude fractionations of this kind, one cannot be certain that the peak is homogeneous.

When the material with the elution characteristics of proinsulin was further purified by coupling with antibody bound to Sepharose and then subjected to gel electrophoresis, a significant proportion of the

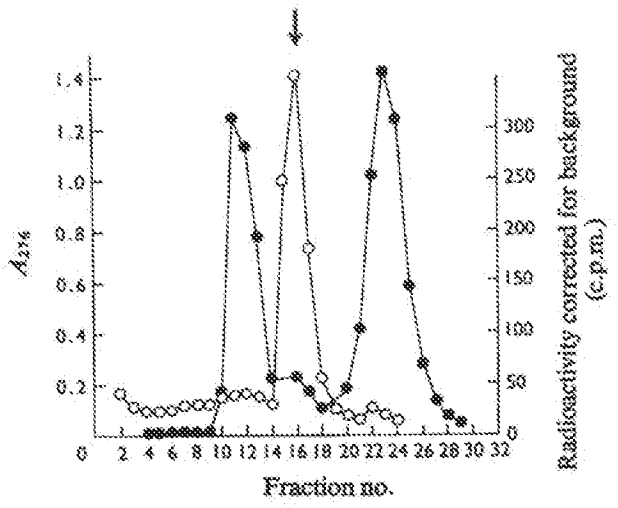

Fig. 1. *Incorporation of* L-*leucine into proinsulin in a broken-cell preparation of islets*

Islets disrupted ultrasonically were incubated in a medium containing [^{3}H]leucine and precipitated with trichloroacetic acid. The precipitated proteins were dissolved in 250 μl of 1 M-acetic acid and applied to a Sephadex G-50 column. Ox insulin (5 mg) was used as a marker (indicated by an arrow). For full details see the Materials and Methods section. ●, Radioactivity corrected for background (c.p.m.); ○, A_{276} for marker insulin.

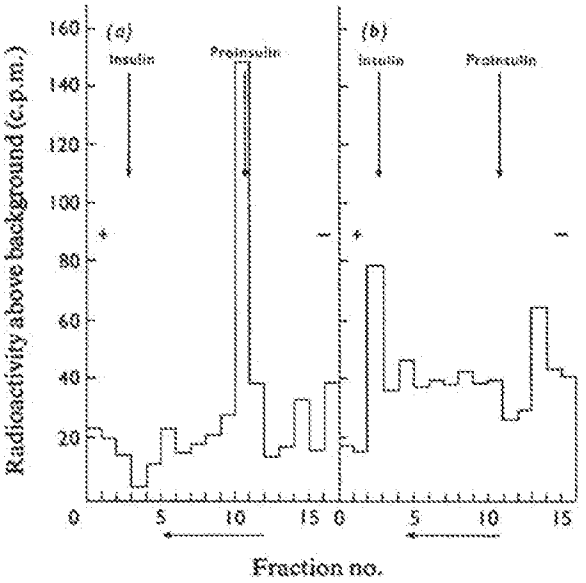

Fig. 2. *Gel electrophoresis of proteins after incubation of a broken-cell preparation of islets with* [^{3}H]*leucine*

Material corresponding to proinsulin from the Sephadex fractionation was treated with anti-insulin antibody bound to Sepharose and extracted with acid/ethanol. After freeze-drying the sample was divided. One sample was left untreated (sample *a*), the other was treated with trypsin (sample *b*). These samples were then subjected to polyacrylamide-gel electrophoresis and the fractions were analysed for radioactivity (for details see the text). In (*a*) 29% of the radioactivity is associated with the proinsulin marker; in (*b*) 21% is associated with insulin and 12% with proinsulin.

radioactivity appeared still to be associated with proinsulin (Fig. 2*a*). When the material was treated with trypsin, radioactivity appeared in a zone corresponding to the ox insulin marker (Fig. 2*b*).

Effect of sugars on incorporation into proinsulin

Raising the concentration of glucose from 2 mM to 16 mM approximately doubled the rate of incorporation into proinsulin by comparison with the effects of 2 mM-glucose, whereas 16 mM-galactose and 16 mM-sucrose caused increases in rate that were not significant (Table 1).

In another series of experiments, cycloheximide (250 μg/ml) significantly decreased the incorporation of [^{3}H]leucine into proinsulin when the homogenate was incubated in 16 mM-glucose. Values obtained were 6.8$\pm$0.9 c.p.m./h per μg of insulin without cycloheximide and 3.1$\pm$0.1 in the presence of the drug and with 16 mM-glucose. This difference is significant ($P<0.001$).

Discussion

Characterization of labelled proinsulin

In a much earlier series of experiments, Wagle (1965) showed that a crude pH 5 fraction of dog pancreas was able to incorporate labelled amino acids into substances that could be precipitated by an antibody to insulin. However, no attempt was made in that work to characterize the product in greater detail. Earlier attempts to incorporate label into subcellular fraction of islets did not result in significant labelling of proteins (Morris & Korner, 1970).

The material made in broken-cell preparations of islets of Langerhans appears to be proinsulin (Figs. 1 and 2). Thus it possesses all the electrophoretic properties of proinsulin and is assumed to cross-react, as would be expected, with an antiserum against ox insulin. Moreover it appears to be converted into insulin by trypsin (Fig. 2*b*). The single peak of proinsulin obtained on gel electrophoresis contrasts with the predominant labelling in insulin that takes place when whole rabbit islets are incubated with labelled amino acids for extended periods (Parry & Taylor, 1974). Other studies (Permutt & Boime, 1975; Weber, 1975) have now suggested that mRNA derived from islets may be translatable in a wheat-germ system leading to the synthesis of a transient

Table 1. *Effects of sugars on incorporation of [^{3}H]leucine into proinsulin in islet-cell homogenates*

The islets were preincubated in 2mM-glucose in a bicarbonate-buffered medium, and after ultrasonic disruption in a Tris/KCl buffer (see the Materials and Methods section), [^{3}H]leucine was added, and the incubation continued for a further 1h. Proteins were precipitated by 10% trichloroacetic acid. The precipitate was dissolved in 1M-acetic acid (see the Materials and Methods section) and the solution was applied to a Sephadex G-50 column, and the proinsulin was further purified by immuno-binding to Sepharose (see the text). Means are shown ± S.E.M. for the numbers of experiments in parentheses. Significance is calculated by using Student's *t* test.

	Addition to medium	Rate of incorporation of [^{3}H]leucine into proinsulin (c.p.m./h per μg)
Broken-cell preparation	2mM-Glucose (10)	4.04 ± 0.57
	16mM-Glucose (10)	7.63 ± 1.00*
	16mM-Galactose (6)	5.31 ± 1.81
	16mM-Sucrose (6)	5.43 ± 0.94
Crude cell-free system	2mM-Glucose (10)	0.37 ± 0.12

* Value significantly different from control with $P < 0.01$.

precursor of proinsulin in a similar way to the production of a parathyrin precursor (Kemper *et al.*, 1974). However, in the present experiments there is no evidence that significant amounts of material closely related to proinsulin, but of higher molecular weight, are being labelled.

It may be concluded that when β-cells are disrupted by ultrasonic methods the enzymic machinery for converting proinsulin into insulin is disorganized, resulting only in the presence of proinsulin, as Kemmler & Steiner (1970) have suggested.

The results obtained could be explained by assuming that incorporation of label takes place in a small number of undamaged β-cells, but this seems unlikely. In the first place labelling is virtually entirely into proinsulin (see Fig. 2) rather than into insulin as in unbroken rabbit islets (Parry & Taylor, 1974). Microscopic examination of the cells, moreover, did not suggest the presence of any intact cells in the preparation, even though routine microscopic checks were made for them in each experiment.

Proinsulin biosynthesis in simple cell-free system of islets

When incorporation of label is studied in a simple cell-free system in which nuclei and cell membrane had been removed by centrifugation, there is still a measurable incorporation of leucine into proinsulin, though this is quantitatively much less than in a broken-cell preparation (Table 1). Because incorporation of label into proinsulin under these circumstances was small and inconsistent, this simple cell-free system did not seem a suitable one for studying the effects of glucose and other stimulating agents on biosynthesis.

Effects of sugars

Increased glucose concentration clearly has an effect on the incorporation of leucine into proinsulin, although the effect is relatively much less than in whole islets, where the difference in response may be up to 10-fold, when there is a change in glucose concentration from 2 to 16mM. This diminished response could be the result of the dilution of a number of intracellular factors required for biosynthesis. Since the effect is blocked by cycloheximide it is clear that proinsulin synthesis *de novo* is being studied.

It is, however, of considerable interest that there appears to be some specificity in the response, since galactose and sucrose, both thought not to be metabolized by islets, are ineffective. The broken-cell preparation therefore resembles whole islets (Lin & Haist, 1975) or pancreas slices (Parry & Taylor, 1966), in which galactose is ineffective in promoting synthesis.

The present experiments indicate that a significant incorporation of labelled leucine takes place into proinsulin in disrupted cells from islets of Langerhans. It follows that the full structural integrity of the β-cell is not necessary for demonstrating the stimulatory effect of glucose on insulin synthesis. It remains to be seen whether or not the glucose effect in broken-cell preparations is due to structures associated with cyclic AMP production, or alternatively to other membrane-associated factors.

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